

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C12Q 1/68</p>	<p>A2</p>	<p>(11) International Publication Number: WO 93/04199 (43) International Publication Date: 4 March 1993 (04.03.93)</p>
<p>(21) International Application Number: PCT/GB92/01526 (22) International Filing Date: 19 August 1992 (19.08.92) (30) Priority data: 9117902.8 20 August 1991 (20.08.91) GB 9202962.8 12 February 1992 (12.02.92) GB (71) Applicant (for all designated States except US): SCIENTIFIC GENERICS LIMITED [GB/GB]; King's Court, Kirkwood Road, Cambridge CB4 2PF (GB). (72) Inventor; and (75) Inventor/Applicant (for US only) : PARTON, Adrian [GB/GB]; 9 Fincham Road, Mildenhall, Suffolk IP28 7BS (GB). (74) Agent: SMART, Peter, John; W.H. Beck, Greener & Co., 7 Stone Buildings, Lincoln's Inn, London WC2A 3SZ (GB).</p>		<p>(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). Published <i>Without international search report and to be republished upon receipt of that report.</i></p>
<p>(54) Title: METHODS OF DETECTING OR QUANTITATING NUCLEIC ACIDS AND OF PRODUCING LABELLED IMMOBILISED NUCLEIC ACIDS</p> <div data-bbox="542 1356 1081 1530" data-label="Chemical-Block"> </div> <p>(57) Abstract</p> <p>Methods for determining the presence or absence of a certain nucleic acid, for producing immobilized nucleic acids and for carrying out cyclic amplification methods like PCR or LCR using immobilized probe-primers are disclosed.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	MN	Mongolia
AU	Australia	FR	France	MR	Mauritania
BB	Barbados	GA	Gabon	MW	Malawi
BE	Belgium	GB	United Kingdom	NL	Netherlands
BF	Burkina Faso	GN	Guinea	NO	Norway
BG	Bulgaria	GR	Greece	NZ	New Zealand
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IE	Ireland	PT	Portugal
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovak Republic
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CS	Czechoslovakia	LU	Luxembourg	SU	Soviet Union
CZ	Czech Republic	MC	Monaco	TD	Chad
DE	Germany	MG	Madagascar	TG	Togo
DK	Denmark	ML	Mali	UA	Ukraine
ES	Spain			US	United States of America

METHODS OF DETECTING OR QUANTITATING NUCLEIC ACIDS AND OF
PRODUCING LABELLED IMMOBILISED NUCLEIC ACIDS

The present invention relates to the detection or
5 quantitation of nucleic acid fragments which are of known base
sequence or which possess a base sequence which is partially
known. In particular, but not exclusively, the present
invention relates to the detection or quantitation of nucleic
acid which is the product of a polymerase chain reaction (PCR)
10 amplification of a sample of DNA. It relates also to the
detection or quantitation of nucleic acid material present at
levels insufficient for detection without amplification
conveniently using known methods. The invention relates also
to the production of labelled immobilised nucleic acids.

15 The known methods of detecting PCR products rely on the
PCR products undergoing some form of purification and/or
separation procedure before they are analysed. One commonly
used method involves the analysis of PCR products by agarose
gel electrophoresis. This separates the amplified DNA
20 fragments and any remaining oligonucleotide primers on the
basis of size. The extent of migration of the PCR products
in the gel can be compared to the distance migrated by a
series of standard oligonucleotide markers of different
lengths. In this way the size of the amplified DNA fragments
25 can be determined. This provides an assessment of whether the
PCR amplification of the sample DNA has taken place. However,
because the PCR products are separated on the basis of size
alone, it is not possible to distinguish between a PCR product
of the desired base sequence and an amplified contaminant of
30 approximately the same length. In order to be sure of the
identity of the PCR products, the agarose gel electrophoresis
is taken one step further and subjected to the Southern
blotting technique (Southern 1975).

In Southern blotting, the DNA fragments in the agarose
35 gel are transferred to the surface of a nylon or
nitrocellulose membrane in direct correspondence to their

relative positions in the gel. A labelled single stranded DNA probe with a base sequence homologous to the sequence of interest is washed over the membrane. The labelled probe hybridises to any polynucleotides or oligonucleotides on the membrane surface which are of a complementary base sequence. The label used for the probe is usually biotin or a radioisotope such as ^{32}P .

Southern blotting is not only able to demonstrate that a PCR product has been generated from the initial sample but it also confirms the identity of that product and so is able to distinguish contaminants which have become amplified. The drawback with Southern blotting is that it is relatively laborious and requires a moderate level of laboratory skill to perform. A certain level of laboratory equipment and facilities are also required. Southern blotting is therefore not particularly suited for use in the rapid screening of a large number of samples or the screening of samples outside the laboratory.

Another known method which can be used to identify PCR products is that of dot blotting. This method uses essentially the same principle as Southern blotting except that no agarose gel electrophoresis is first performed to separate the components of the samples. Instead, the PCR products are spotted directly onto a membrane, washed, and then hybridised with a labelled DNA probe of complementary base sequence. Dot blotting is less informative than Southern blotting and it is unable to differentiate the desired PCR products from contaminants on a size basis. The advantage over Southern blotting is that it is somewhat quicker, but even so dot blotting still suffers from the disadvantages of being relatively labour intensive and is time consuming.

WO89/11546 describes a method of both amplifying and analysing a particular DNA sequence. The method involves conducting PCR on a sample of DNA in which one of the primer sequences is linked to magnetic particles. The other primer sequence remains free in solution. When standard PCR is

performed on a sample using the particle bound primers and the free primers, amplified products become formed on the magnetic particles. The magnetic particles are then easily recovered for analysis by using a magnet which serves to draw the magnetic particles out of the PCR mixture. The processes of sample fragment capture, its amplification and subsequent analysis are performed sequentially using the magnetic particles.

In WO 89/11546, analysis of the particle bound amplified sequence after PCR can be performed in a variety of ways. For example, a labelled single stranded DNA probe of complementary base sequence to that of interest can be hybridised to the particles. The particles can then be removed with a magnet, washed and then the amount of label hybridised to the amplified strand determined.

A second way of analysing the particle bound amplified PCR products is to provide a complementary primer sequence, labelled deoxynucleoside triphosphates and DNA polymerase. Any hybridisation of the primer to the amplified strands will result in the formation of a labelled complementary strand hybridised to the amplified particle-bound strand. Again the particles can be removed, washed and the amount of label associated with them determined. Alternatively, double stranded DNA is produced by providing deoxynucleoside triphosphates, DNA polymerase and an oligonucleotide probe to the particle bound amplified single stranded DNA. The particle bound amplified single stranded DNA will serve as a template and the synthesis of a complementary strand will take place. Double stranded DNA is then demonstrated by a biotinylated component which intercalates non-specifically with dsDNA.

The teaching of WO 89/11546 if it is operable at all has the drawback that contaminant DNA fragments may all too easily be picked up by the particle bound primers and amplified. There is therefore no selectivity exercised against contaminant sequences becoming bound to the particles and then

being amplified by PCR. The discrimination between contaminants and genuine PCR products is left to the final analysis stage and therefore depends entirely on the specificity of the labelled probe, or the further primer
5 sequence for the DNA sequence of interest which is used to screen the magnetic particles. Furthermore, the only form of substrate to which the primer can be immobilised is magnetic beads. The process cannot be adapted to use primers immobilised to more convenient substrates such as solid
10 surfaces.

Furthermore, the process involves hybridisation and or polymerase reaction steps after the PCR reaction proper, followed by determination of the thus bound label. Most seriously we have appreciated that the label is never bound
15 covalently to the magnetic beads, so that one cannot employ stringent conditions to remove all non-specifically bound labels from the beads because of the risk of dehybridising and losing labelled DNA.

It would be desirable to develop methods of the kind indicated above in which some or all of these drawbacks are
20 avoided.

We have appreciated that it is possible to improve in both the rapidity and specificity involved in demonstrating the presence of a particular nucleic acid sequence in a
25 sample. This is done by test method which involves simultaneously extending and labelling by polymerase action an immobilised single stranded probe-primer of complementary base sequence to the sequence of interest. The extension and labelling of the immobilised probe-primer will be brought
30 about when a strand of nucleic acid of complementary base sequence hybridises to the probe-primer and extends beyond the 3' end of the probe-primer to provide a single stranded template.

Accordingly, the present invention provides a method for
35 establishing the presence or absence in a sample of a nucleic acid containing a target nucleotide base sequence or

-5-

quantitating the presence of said nucleic acid containing the target nucleotide base sequence, comprising the steps of:

- 5 (a) exposing a sample of nucleic acid to an immobilised nucleic acid probe-primer, at least a portion of the probe-primer being capable of hybridisation with the target sequence if present in said sample under conditions such as to allow the target sequence if present to hybridise with the immobilised probe-primer;
- 10 (b) (i) subjecting any probe-primer/target sequence hybrids so formed to a polymerase reaction in the presence of at least one labelled deoxynucleoside triphosphate substrate so as to extend and label the probe-primer; or
- 15 (b) (ii) subjecting any probe-primer/target sequence hybrids so formed to hybridisation of a portion of said target sequence adjacent the portion hybridised to said probe/primer, said hybridisation being with a labelled second oligonucleotide primer complementary in sequence to said adjacent portion
- 20 of the target sequence, and ligating said probe-primer and second primer; and
- (c) detecting or quantitating any label or amount of label bound to the extended immobilised probe-primer.
- 25

Prior to said detection step one will generally need to separate the immobilised probe-primer from the sample and any unreacted deoxynucleoside triphosphate substrates.

30 In an alternative aspect the invention provides a method of producing immobilised labelled nucleic acid comprising the steps of:

- (a) exposing a nucleic acid containing a target nucleotide base sequence to an immobilised nucleic acid probe-primer under hybridising conditions, at least a portion of the probe-primer being capable
- 35 of hybridisation with the target base sequence, so

- as to allow nucleic acid containing the target base sequence to hybridise with the probe-primer; and
- (b) (i) subjecting the hybridised probe-primer to a polymerase reaction in the presence of at least one labelled deoxynucleoside triphosphate substrate so as to extend and label the probe-primer; or
- (b) (ii) subjecting any probe-primer/target sequence hybrids so formed to hybridisation of a portion of said target sequence adjacent the portion hybridised to said probe-primer, said hybridisation being with a labelled second oligonucleotide primer complementary in sequence to said adjacent portion of the target sequence, and ligating said probe/primer and second primer.
- Generally, it is envisaged that the purpose of producing such labelled nucleic acids will be for the purpose of demonstrating the presence in a sample of the nucleic acid sequence which acts as a template for the extension of the probe-primer.
- After the polymerase reaction, the immobilised probe-primer may be separated from the nucleic acid containing the target sequence and labelled deoxynucleoside triphosphates. Most conveniently, this may be done by removing the substrate carrying the immobilised probe-primer and its extension products from the reaction mixture. The substrate may be removed under denaturing conditions so that the immobilised extended probe-primer is in single stranded form. Alternatively, the substrate may be removed under hybridising conditions and subjected to denaturation conditions. The substrate bearing the immobilised extended probe-primer may be washed to remove other reaction mixture constituents. In particular, the substrate with the immobilised extended probe-primer may be subjected to washing conditions sufficiently vigorous to remove all products other than the immobilised extended probe-primer and any remaining unextended probe-primer from the substrate. As is described in more detail

hereafter, the probe-primer is preferably immobilised to the substrate by covalent bonding so that the washing conditions may be sufficiently vigorous to ensure the denaturation and removal of all nucleic acid materials from the substrate other than the covalently bonded probe-primer and its extension products, thus ensuring that subsequent detection of the extension products is not subject to interference from non-specifically bound nucleic acid materials.

Preferably, the polymerase reaction is carried out in the presence of denatured nucleic acid complementary in sequence to said nucleic acid containing said target sequence so that the target sequence may be present in one strand of an initially double-stranded nucleic acid, both strands of which are present in the reaction mixture.

The nucleic acid may be DNA and the reaction is then carried out under the influence of a suitable DNA polymerase.

Alternatively, the nucleic acid may be an RNA and the reaction may then be carried out under the influence of an RNA dependant DNA polymerase (reverse transcriptase).

The nucleic acid containing the target sequence may be amplified in the presence of or prior to the introduction of the immobilised probe-primer. Thus, the nucleic acid containing the target sequence may be amplified by hybridisation of the complementary strand to a probe oligonucleotide at a location which lies in the 3' direction from the 3' end of the sequence of the complementary strand which is complementary to the target sequence to which the immobilised probe-primer hybridises. The use of a polymerase will extend the primers to complete the target sequence to which the immobilised probe-primer will hybridise upon proceeding through denaturing conditions and hybridising conditions.

Whilst it is possible to produce some amplification using a single primer, preferably according to the method of the invention during said polymerase reaction, two oligonucleotide primers are provided, the first primer being capable of

hybridisation to the complementary strand at a location which lies in the 3' direction from the 3' end of the sequence of the complementary strand which is complementary to the target sequence to which the immobilised probe-primer hybridises, the
5 second primer being capable of hybridisation with said target sequence containing nucleic acid at a location which lies in the 3' direction from the 5' end of the target sequence to which the probe-primer hybridises, and the first primer having no sequence homology with the immobilised probe-primer or the
10 second primer sufficient to produce hybridisation therebetween.

Thus, it is possible to conduct a polymerase chain reaction to amplify a target sequence of interest which will then be detected by hybridisation with the probe-primer and
15 extension of the probe-primer with labelled nucleotides.

Preferably, the probe-primer is longer than the second primer so that the specificity of hybridisation between the probe-primer and the target sequence is substantially greater than the specificity of binding of the second primer under
20 similar conditions. Thus, the method according to the invention can be used not merely to ascertain that extension of the second primer has taken place but also to confirm that the product of the extension is the expected product.

Whilst the first and the second primers may typically be
25 from 7 to 25 nucleotides in length, it is preferred that the probe-primer is greater than 15 nucleotide bases long, for example the probe-primer may be from 20 to 40 bases long or longer, e.g up to 100 or several hundred base pairs.

It is not essential that either the first and second
30 primers or the immobilised probe-primer are exactly complementary in sequence to the sequences to which they are required to hybridise in the reaction. It is sufficient that they are sufficiently complementary for hybridisation to occur to a sufficient extent for the polymerase reaction to proceed.

35 The denatured nucleic acid exposed to the immobilised probe-primer may be a product of the polymerase chain reaction

(PCR) process or may be a product of the ligase chain reaction (LCR) process described in published European Patent Specification No. 320308-A2. The PCR process used need not necessarily be the "standard" PCR process where two
5 oligonucleotide primers are used which each recognise different sequences either side of the target sequence. "Anchored" or "inverse" PCR may be used where it is only necessary to have a single primer of known sequence.

At least one amplification cycle of the PCR process is
10 preferably conducted in the presence of the said probe-primer.

The probe-primer may be present from the outset of the PCR process or may be introduced for one or several cycles toward the end of the PCR process. Alternatively, it may be present at the outset and may be removed and analysed before
15 proceeding with several further cycles having once confirmed that the desired product is being produced.

At least one amplification cycle of the LCR process may be conducted in the presence of the said probe-primer. The probe-primer may be present from the outset of the LCR process or may be introduced for one or several cycles towards the end of the LCR process. The polymerase mediated extension labelling of the probe-primer may be performed in the presence of the LCR reaction components. Alternatively, the probe-primer may be removed from LCR reaction components before
20 subjecting it to polymerase reaction in the presence of deoxynucleoside triphosphates and a labelled deoxynucleoside triphosphate.

The product of a PCR reaction, or more preferably the product of an LCR reaction can also be detected by hybridising
30 the product to an immobilised probe primer, as described, separating the immobilised probe primer/target sequence hybrids from the reaction mixture, adding a labelled oligonucleotide second primer chosen to hybridise to the target sequence adjacent the hybridisation of the probe-primer
35 to the labelled oligonucleotide, and ligating the probe-primer to the second primer.

-10-

The second primer should be sufficiently close to the probe-primer for ligation to operate. The second primer can be provided as a single primer or as a series of two or more primers to hybridise adjacent one another on the target sequence so as to be ligatable to one another and the probe-primer. The label need not then be on the component of the second primer nearest to the probe-primer.

The polymerase used may be any suitable polymerase as known in the art including Taq polymerase, DNA polymerase I, DNA polymerase Klenow fragment, or T4 polymerase.

In order to more intensively label the bound nucleic acid probe primer, cycles of probe primer extension, denaturation of extended primer/sample nucleic acid hybrids, and rehybridisation of sample nucleic acid (whether itself the product of an amplification procedure or not) to further bound probe primer sites may be performed. By such a procedure, each molecule of sample nucleic acid may be used to produce labelling of as many sites on the solid support as there are cycles preformed. This produces a substantially linear amplification of the labelling of the probe-primer on the solid support without amplification or without further amplification of the sample or target nucleic acid.

Such a method may be employed for the direct detection (i.e. without target amplification) of nucleic acid, e.g. from a sample suspected of containing a micro-organism.

Methods according to the invention may also be used for such direct detection of nucleic acids without cycling procedure provided that the number of sample nucleic acid molecules is sufficient. Micro-organisms have a large number of identical RNA molecules per organism. Thus, methods according to the invention can be used for the detection or quantitation of a specific micro-organism in a sample without preliminary amplification of the target nucleic acid, by choosing as said target nucleic acid RNA present at a sufficient level in each organism cell.

-11-

To facilitate the presentation of the sample nucleic acid to the immobilised probe primer, one may use affinity capture, preferably employing monoclonal antibodies on a solid support as a capture agent. Micro-organism cells captured in this way may be lysed to release nucleic acid for hybridisation to said immobilised probe primer, followed by probe primer extension. Suitable methods for immobilising monoclonal antibodies for use in affinity capture are well known in the art.

Preferably, the immobilised probe-primer is covalently linked to a solid support in such a way as to leave the 3' end of the nucleic acid available for extension. However, the invention may be practised using nucleic acid immobilised to a support other than by binding of the 5' end provided that sufficient free 3' ends are available for extension. Thus, random binding of the probe-primer to the support may be adequate depending upon the circumstances.

Several methods are known in the art for immobilising nucleic acids. These include methods which involve the modification of the 5' phosphate of the nucleic acid as a preliminary stage. Thus it is known to provide a 5' NH_2 modified DNA which may then be bound to a hydroxyl group presenting surface that has been activated by reaction with cyanogen bromide (Clerici *et al* (1979) Nucl. Acids Res. 6: 246-258). Similarly modified DNA may be reacted with a surface having carboxyl groups activated by reaction with 1-ethyl-3-(3-dimethylpropyl)carbodiimide (EDC). (Gosh *et al* (1987) Nucl. Acids Res. 15: 5353-5372). The 5' phosphate groups of DNA can also be activated to react with hydroxy or amine groups on a support by reaction with carbodiimides. Thus 5' phosphate groups activated by reaction with 1-cyclohexyl-3-(2 morpholinomethyl) carbodiimide (CMC) can be reacted with a substrate presenting hydroxy or amine surface groups (Gilham P.T. (1968) Biochemistry 7: 2809-2813). DNA activated by reaction of its 5' phosphate groups with EDC will bind to a substrate presenting surface amine groups. Binding of DNA to substrates randomly along the length of the DNA can

-12-

be achieved by modifying an hydroxy group presenting substrate to present aromatic primary amine groups which can be diazotised and reacted with bases of the DNA. Similarly cyanogen bromide activated, hydroxy group presenting substrates can be reacted with DNA bases.

However, it is preferred according to the invention to achieve 5' specific bonding of nucleic acid probe-primers to a substrate presenting carboxyl groups by activating the surface with an activating agent which is preferably a carbodiimide to make the carboxyl groups readily but selectively reactive with the bases exposed in a single stranded portion of a double-stranded nucleic acid. The single stranded portion may be at the 5' end of the nucleic acid or at an intermediate portion along the length of the nucleic acid.

The substrate may be activated by treatment with a solution of carbodiimides such as CMC or EDC. Generally, the reaction conditions are not critical. Temperatures of about 50°C are preferred but lower temperatures can be used with longer reaction times. A pH of about 7 is preferred but more acidic pH will be acceptable. A carbodiimide concentration of 0.05 to 0.5 molar may be used, preferably about 0.1 molar. Reaction is suitably continued until the reactivity of the surface is maximised, e.g. for about 3 hours, at 50°C, using a carbodiimide concentration of 0.1 M and pH 7.

The immobilisation of the nucleic acid probe-primer with the activated substrate may be carried out simply by contacting a solution of the partially single stranded nucleic acid with the activated surface for a sufficient period. The temperature is preferably slightly elevated but should not be such as to produce partial denaturing. A temperature of from 30 to 60°C e.g. 50°C is preferred. A reaction time of about 3 hours will generally be adequate.

The substrate may be a nylon, e.g. Nylon 66. Other methods for reacting carboxyl or other groups on the substrate selectively with nucleic acid bases present in a single-

-13-

stranded portion of a double-stranded nucleic acid may be employed. For instance, the substrate may be reacted with a linker compound having a first group reactive with the surface and a second group reactive (optionally after activation) with the nucleic acid bases so as to form a surface which can be reacted (possibly after a further activation step) with the nucleic acid to be immobilised.

The linker compound may for instance be such as to convert the hydroxy group presenting surface into a carboxyl group presenting surface for activation with carbodiimide. Alternatively, it may be a compound reactive with carboxyl or amine groups on a substrate and with nucleic acid bases, e.g. a quinone such as para-benzoquinone.

DNA having short single-stranded portions can be produced by the use of restriction enzymes to produce restriction fragments having sticky ends. Alternatively, single-stranded DNA's of complementary sequences but different lengths may be annealed together to leave a single-stranded portion at the 5' end, the 3' end, or both ends as preferred. Two shorter strands may be annealed to one longer strand to leave a single-stranded portion between them. The position of the single-stranded portion or portions will determine the position of the attachment and orientation of the immobilised nucleic acid.

Once immobilised the nucleic acid may be denatured to produce single-stranded nucleic acid immobilised on the substrate for use as a probe-primer according to the present invention.

One or more of the deoxynucleoside triphosphate substrates present in the polymerase reaction may be labelled. The label may be of any type detectable by a subsequent assay. For instance, the label may be a radioisotope, an enzyme capable of catalysing an assay reaction, a fluorescent label, an antigen or antibody or may be biotin.

The invention includes a method of detecting a target nucleic acid sequence comprising the polymerase or ligase

-14-

mediated extension and labelling of an immobilised probe-primer sequence complementary to at least a part of the target sequence in the presence of products of a polymerase chain reaction (PCR) or a ligase chain reaction (LCR), at least a
5 portion of the base sequence of the said PCR or LCR products containing the target sequence.

In a preferred aspect, the invention provides a method for detecting a presence in a sample of copies of target nucleic acid sequence generated by a polymerase chain reaction
10 process comprising:

- (a) exposing an immobilised probe-primer sequence complementary to at least part of the target sequence to a denatured sample under hybridising conditions;
- 15 (b) subjecting the immobilised probe-primer to a polymerase reaction in the presence of at least one labelled deoxynucleoside triphosphate substrate so that the immobilised probe-primer becomes extended and labelled when hybridised to a portion of the
20 target sequence; and
- (c) detecting any label bound to the immobilised probe-primer.

In a further preferred aspect the invention provides a method for detecting the presence in a sample of copies of a
25 target nucleic acid sequence generated by a polymerase chain-reaction process employing a first primer and second primer comprising:

- (a) conducting at least one PCR cycle in the presence of an immobilised probe-primer sequence
30 complementary to at least part of the target sequence in addition to said first and second PCR primers so that the immobilised probe-primer becomes extended when hybridised to a portion of the target sequence; and
- 35 (b) detecting the presence of the extended immobilised probe-primer.

-15-

Preferably, at least one PCR cycle is carried out in the presence of at least one labelled deoxynucleoside triphosphate substrate so that the probe-primer becomes labelled as it is extended.

5 An alternative option however is to detect the extension of the immobilised probe-primer by other means. Thus, the method may comprise in step (b) denaturing extension products of the probe-primer and hybridising to the bound extension products a labelled probe which may thereafter be detected in
10 a known manner.

The invention includes the use of a nucleic acid probe-primer immobilised to a solid support for hybridisation and extension in a polymerase reaction utilising at least one labelled nucleotide.

15 The invention further includes a kit for performing a method according to any one of the various aspects of the invention described above comprising a nucleic acid probe-primer immobilised to a solid support such that the 3' end of the probe-primer is available for polymerase extension and one
20 or more of; a DNA polymerase enzyme, appropriate deoxynucleoside triphosphate substrates, at least one of which is labelled, or one or more oligonucleotide primers for amplification of a target substrate for the probe-primer.

The invention will now be further described and
25 illustrated with reference to the accompanying drawings and general and specific examples.

In the drawings:

Figure 1 shows schematically the nucleic acid species present at the start of a typical PCR cycle in accordance with
30 preferred aspects of the invention.

Figure 2 shows an immobilised probe-primer sequence characteristic of the invention linked by its 5' end to a substrate.

Figure 3 shows a single strand of sample DNA with a
35 portion of complementary base sequence hybridised to the

-16-

immobilised probe-primer. The unhybridised portion of the sample strand can act as a template.

Figure 4 shows how DNA polymerase acts to extend the immobilised probe-primer using the single stranded portion of the hybridised DNA strand.

Figure 5 shows the resulting extended and labelled immobilised probe-primer linked to the detector.

Figure 6 illustrates schematically a process according to the invention in which the level of labelling obtained is amplified by a cyclic process.

In preferred aspect the present invention may be used for indicating the presence of a particular DNA sequence in a sample when the particular DNA sequence is present only in low copy number. The sample is amplified by PCR in the presence of a detector comprising an immobilised probe primer. A first embodiment in accordance with the invention is shown schematically in the Figures. A detector comprises a Nylon 66 substrate and has a single stranded oligonucleotide probe-primer of the desired sequence covalently linked to the substrate. The probe-primer is linked to the substrate by its 5' end leaving pendant single stranded molecules available for hybridisation. The linkage is achieved by activating the carboxyl groups of the substrate with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CMC). The activated detector is then reacted with a double stranded nucleic acid with a single stranded portion. Binding takes place between the amino groups on the bases of the single stranded portion and the modified carboxyl groups. The bound nucleic acid is then denatured to remove the unbound strand from the detector. The probe-primer sequence is by way of example 50 nucleotide bases long (schematically reduced to 8 bases in Figure 1) and is complementary to a portion of the 3' end of the strand of the particular DNA sequence to be detected.

A preferred method in accordance with the invention utilising the detector described above by way of general example is as follows and is shown in Figures 1 to 5. A

-17-

sample containing a DNA sequence of interest and a complementary strand (species 7 in Figure 1) comprising a target strand (species 6 in Figure 1) is added to a reaction tube together with Taq polymerase, two selected oligonucleotide short primers each of about 20 nucleotide bases and deoxynucleoside triphosphate substrates; deoxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP) and deoxyguanine triphosphate (dGTP). Biotin-labelled deoxyadenosine triphosphate (biotin-dATP) is included so that the PCR products incorporating the biotin-dATP become labelled with biotin.

The first short primer has a base sequence which is neither the same as nor complementary to the probe primer sequence. The first short primer sequence is selected so as to be capable of hybridisation with the complementary single strand of the DNA sequence of interest which does not hybridise to the probe-primer. The hybridisation location for the first primer lies in the 3' direction from the 3' end (downstream) of the sequence AAAATGCT (SEQ ID:1) of the complementary strand which sequence is complementary to the target sequence to which the immobilised probe-primer hybridises. Thus the first probe GGGG (SEQ ID:2) will not hybridise to the probe-primer. The second short primer has a base sequence which is the same as a portion of the base sequence of the probe-primer and so is therefore able to hybridise with the same single strand of DNA as the probe-primer.

However, the second primer may not correspond to any part of the probe-primer but may be complementary to any region of the PCR product strand 1 which lies in the 3' direction of the 5' end (A) (downstream) of the target sequence to which the probe-primer hybridises, e.g. in the 3' direction of the 3' end (T) (downstream) of the said target sequence. Thus the probe-primer may have as its target a sequence in the PCR product strand 1 (species 2 in Figure 1) which does not lie right at the 3' end, although it is preferred that this target

-18-

sequence at least lies close to the 3' end of the target sequence.

The detector is placed in a reaction tube with the sample and reaction components and a polymerase chain reaction is conducted for preferably 20 to 30 amplification cycles of heating and cooling. The level of PCR products in the sample increases exponentially with each amplification cycle that takes place. During the course of PCR, as sufficient PCR products become available, preferential hybridisation over the second short primer takes place between the probe-primer and PCR products which possess base sequence homology. Preferential hybridisation takes place because the longer probe-primer has a greater specificity for the base sequence of interest than the second short primer. Because the probe-primer is single stranded and linked by its 5' ends to the detector, the 3' end of a homologous single stranded PCR product hybridises with the probe-primer so that its 5' end remains single stranded and thereby acts as a template for a suitable polymerase. Synthesis of a complementary strand from the template is initiated from the 3' end of the hybridised probe-primer and takes place in the 5' to 3' direction until the end of the template is reached. The action of the polymerase extends the 3' end of hybridised probe-primer and in doing so causes it to become labelled with biotin due to the incorporation of biotin-dATP residues.

When PCR has been completed, the detector is removed from the PCR mixture and sample. Removal may take place under denaturing conditions at an elevated temperature so that the probe-primer is not hybridised with PCR products with base sequence homology. The detector is then washed in several changes of buffer. This washing step removes any non-specifically bound PCR products, primers or deoxynucleoside triphosphate substrates from the tube. Once washing has been completed then the presence of any biotin label bound to the detector tube can be determined by incubation with an avidin-peroxidase enzyme complex. After washing away any unbound

-19-

avidin-peroxidase complex the peroxidase enzyme is provided with a suitable substrate such as 2,2'-azido-bis(3-ethyl-benzthiozoline-6-sulphonic acid). If any peroxidase has been bound to the detector then a readily detectable coloured product will be formed. The term "avidin" as used herein includes "streptavidin" and other avidin like materials.

The method described above used in conjunction with the detector can be modified in any one of the following ways:

- (a) the detector can be placed in a sample and PCR mixture before the start of sample amplification. The detector is kept in the sample and allowed to participate in PCR for fewer than the total number of amplification cycles of denaturation and hybridisation used. The detector can then be removed and analysed for biotin label. The sample amplification by PCR can then optionally be continued without the detector;
- (b) DNA of the sample can be amplified by PCR in the absence of any detector. Once PCR has been conducted for a sufficient number of cycles the detector is added and allowed to participate in just one amplification cycle of denaturation and hybridisation. The detector is then removed at an elevated temperature, by washing and subjected to an analysis for the amount of biotin label;
- (c) a DNA sample can be amplified by PCR for a desired number of amplification cycles of denaturation and hybridisation. The detector can then be added and PCR continued for a further series of amplification cycles of denaturation and hybridisation.

Instead of removing the detector from the sample and PCR mixture under denaturing conditions, or after its removal, subjecting the detector to denaturing conditions, the detector can be removed under hybridising conditions so that PCR products are hybridised to the probe-primer. This has the effect of increasing the quantity of label bound to the detector and therefore increases the final signal intensity.

-20-

The detector in accordance with the second aspect of the invention can be made in a wide variety of physical forms. For example, rods, rods with flattened ends, sheets, sticks or tubes or other containers. The detector can be in the form of a plurality of beads, e.g. small spherical beads which may be magnetic.

The detector may take the form of a reaction tube with the probe-primers linked to the inside wall of the tube. The linkage may be directly with the tube itself or via a carrier sheet, e.g. of nylon, adhered to the tube wall. The detector may be in the form of a microplate having a plurality of wells.

When the detector is of the "dip-in" variety in accordance with the second aspect of the invention, suitably shaped handling portions can be provided in order to avoid a user touching the region of the detector carrying the probe-primers.

Further modifications of the detector can allow for simultaneous detection of a number of different DNA base sequences in the same sample. Different oligonucleotide probe-primers can be linked to separate portions of the detector, e.g. sectors of a detector tube as described above or quadrants of a portion of a detector strip as described above.

The biotin-avidin detection system used need not be a peroxidase enzyme. Other avidin-enzyme complexes may be used.

Of the avidin-fluorescent compound complexes, avidin linked to fluorescein isothiocyanate (avidin-FITC) or avidin linked to rhodamine are suitable. Detection of label on the detector is readily achieved in the case of fluorescent labels by viewing the detector under a light source of appropriate excitation wavelength.

As an alternative to a biotin-avidin detection system, radiolabelled deoxynucleoside triphosphate substrates can be included in the PCR. In this way the probe-primer extensions resulting from homologous PCR product hybridisation will

-21-

become radiolabelled. A suitable radioisotope for this purpose is ^{32}P . The amount of ^{32}P bound to the detector can readily be determined by scintillation counting or other known methods.

5 As well as simply detecting the presence of labelled nucleotide bases in the probe-primer extensions, certain methods in accordance with the present invention can be used in a quantitative fashion. By appropriate adjustment of the DNA sample concentration and the conditions of exposure to the
10 DNA polymerase, the detection of DNA in samples can be conducted in order to obtain quantitative determinations. The extent of labelling of the probe-primers resulting from hybridisation with homologous DNA sequences can be compared to a series of standard determinations to provide a measure
15 of the number of copies of homologous DNA sequence in a sample. The amount of labelling for example will be reflected in the colour density of enzyme catalysed product, the degree of fluorescence, or by the amount of radioactivity depending on which particular labelling system is used.

20 Furthermore, when simultaneous detection of more than one DNA sequence is undertaken, the detection can be performed in a quantitative fashion in order to obtain the relative abundances of the DNA sequences in question.

The method of the present invention is particularly
25 suited to automation. In the case where a detector tube is used, the detection process can be integrated with an automated PCR process. Significant opportunities exist with this aspect of the invention for rapid determination of DNA in samples in a single machine.

30 The invention is not restricted to the detection of samples which are amplified by PCR. For example, detectors of the present invention can be used on samples amplified by the ligase chain reaction (LCR) described in EP A2-0320308. Detectors can be present during LCR but because of the
35 presence of a number of complementary pairs of LCR probe sequences which cover the entire sequence to be amplified, the

-22-

base sequence of the immobilised probe-primer preferably does not have sequence homology with any more than a 10 base length of any LCR probe. In this way the LCR probes will be less likely to hybridise with the immobilised probe-primer in preference to their target strand.

5 Detectors of the present invention can be used in many applications where the detection of specific DNA sequence in a sample is desired. In such methods the sample should already contain sufficiently high copy number of the sequence in question for hybridisation to take place to the probe-primer to a detectable extent. There is no particular need for the copy number to saturate the available number of probe-primers on the detector. In the above mentioned methods the DNA strands of the sample are denatured, e.g. by heating, in order to separate the DNA of the sample into single strands. An enzymatic method of denaturation could be used. The denatured single stranded DNA is then exposed to the detector followed by treatment e.g. cooling in order to permit hybridisation to take place with the probe-primers. A DNA polymerase enzyme and deoxynucleoside triphosphate substrates are then added to the detector so that any probe-primers having a hybridised DNA fragment become extended by the action of the DNA polymerase, the hybridised single strand DNA fragment acting as a template strand.

20 A further modification of the method of the invention is to detect PCR or LCR products using any of the detectors described above but having separated the PCR or LCR product from the PCR or LCR reaction components first. The PCR or LCR product can be separated by precipitation with alcohol for example or by passing it through a gel-filtration column. The detector is then used with the purified PCR product which is denatured into single strands. After hybridisation to the detector, the DNA polymerase is added, together with the deoxynucleoside triphosphate substrates, one of which is labelled e.g. with biotin. The action of the DNA polymerase will extend the probe-primer when it is hybridised to a

-23-

portion of single stranded PCR or LCR product to act as a template for extension of the probe-primer from its 3' end. No cycles of denaturation and hybridisation are required, just a single reaction with a DNA polymerase and the deoxy-nucleoside triphosphate substrates.

Figure 1 may also be used to illustrate the direct detection (i.e. without prior amplification) of a sample nucleic acid which may for instance have been lysed from a micro-organism cell in a sample. The nucleic acid molecule (which is preferably RNA) is captured by the probe primer, which is then extended and labelled by a polymerase reaction (using a reverse transcriptase if the sample is RNA).

Optionally, as shown in Figure 6, the intensity of labelling obtained is increased by the further cyclic steps of dehybridising the target nucleic acid sequences from the extended probe primer, e.g. by heating, rehybridising it to a further probe primer molecule on the solid support repeating the extension-labelling procedure. This may be repeated through as many cycles as desired, e.g. 2 to 40 cycles, preferably 5 to 15 cycles.

Example 1

Surface labelling during PCR

In this example, a sample is tested for the presence of copies of the HlyA gene from Listeria monocytogenes. The base sequence of this gene is known (Mengaud et al. Infect. Immun. 56: 766-772 (1988)). A surface immobilised probe-primer is constructed to detect copies of the gene. Primers are also constructed to perform PCR on the sample. Extension labelling of the immobilised probe-primer indicates the presence of copies of the gene in the sample. The sensitivity of detection is enhanced by amplifying any gene copies present in the sample by PCR. PCR is conducted in the presence of the immobilised probe-primer as follows.

-24-

An oligonucleotide probe-primer with a base sequence homologous to a portion of the Listeria monocytogenes HlyA gene is prepared by a standard method of oligonucleotide synthesis. The probe-primer is 21 nucleotide bases long and has the following base sequence which is complementary to residues 778 to 798 of the HlyA gene

```

              778                      798
              |                        |
10          5'TTTTACGTTCTTCTTCAGTAA3'      (SEQ ID:3)

```

Alternatively, one may use, e.g. a 41-mer, in which a poly C tail is added at the 5' end, thus:-

```

15  5' - CCCCCCCCCCCCCCCCCCCTTTTACGTTCTTCTTCAGTAA 3' (SEQ ID:4)

```

The reason for adding the homopolymeric chain of 20 cytosine residues is to ensure that the capture probe is "free" in solution, i.e. spaced away from the solid support in use.

In order to attach the probe primer covalently by its 5' end to the nylon membrane it is first made double stranded except for the two 5' end deoxycytosine residues. To do this a complementary single strand of DNA 39 nucleotide bases long is synthesised and then hybridised with the 41 nucleotide base long probe-primer to produce;

ds Probe-primer:

```

30  5'CCCCCCCCCCCCCCCCCCTTTTACGTTCTTCTTCAGTAA3' (SEQ ID:5)
    3'GGGGGGGGGGGGGGGGGGGAAAATGCAAGAAGAAGTCATT5'

```

A strip of nylon 66 selected as a substrate is treated with a solution of 0.1M CMC and 10mM Tris-HCl at pH 7.0 for 3 hours at 50°C. The strip is washed 5 times for 5 minutes each time in 10mM Tris-HCl pH 7.0 buffer solution. Surface

-25-

carboxyl groups of the substrate are thereby activated.

A solution of the synthesised probe-primer double stranded DNA in 10mM Tris-HCl buffer solution is applied to the strip. The substrate is left in contact with the DNA solution at 50°C for 3 hours to immobilise the DNA.

The immobilised DNA is denatured by heating and washed in the buffer solution at 90°C.

Two oligonucleotide primers are synthesised using standard methods. The sequence of primer A is the same as the base sequence of the HlyA gene between and inclusive of residues 758-777;

Primer A: 5'TCGGCGCAATCAGTGAAGGG3' (SEQ ID:6)

Primer B is complementary to the base sequence of the HlyA gene between and inclusive of residues 882-901 and has the sequence;

Primer B: 3'CGTCAACGTTTCGCGAACCTC5' (SEQ ID:7)

The following stock reagents are made up from their components or obtained from commercial sources:

10x Taq polymerase buffer; Primer A 0.1 mg.ml⁻¹; Primer B 0.1 mg.ml⁻¹; 10x nucleotide mix - 2 mM each of dGTP, dATP, dCTP and 1.5 mM of dUTP; 0.5 mM Biotin-11-dUTP; and Taq polymerase 3 x 10³ units. ml⁻¹.

The nylon membrane carrying the immobilised probe-primer is placed in an eppendorf tube. The following additions were made:

Taq polymerase buffer stock	10µl
Primer A	5µl
Primer B	5µl
Nucleotide mix	10µl
Biotin-11-dUTP	10µl
Taq polymerase	1µl

-26-

Sterile distilled water	49µl
Sample	10µl
	<hr/>
	100µl

5

The eppendorf tube and its contents are subjected to a period of thermal cycling in a commercially available microcomputer controlled heating block. Each cycle consists of denaturation at 95°C for 1 minute, annealing at 50°C for 30 sec and extension at 72°C for 1.5 minutes. After the 30 cycles the nylon membrane is removed from the eppendorf and washed in two changes of 10 mM Tris-HCl pH 7.0 buffer at 95°C to remove any unbound amplified DNA or incorporated nucleotides. The nylon membrane is then subjected to a standard colorimetric detection procedure for biotin using avidin linked to alkaline phosphatase. Addition of the substrate 2,2'-azido-bis(3-ethyl benzthiozoline-6-sulphonic acid) caused the membrane to become coloured indicating that the avidin carrying the alkaline phosphatase enzyme is immobilised to the membrane by binding to biotin. The presence of the biotin shows that there has been extension of the membrane bound probe-primer by the Taq polymerase.

25

Example 2

Surface labelling after sample amplification by ligase chain reaction (LCR)

In this example any copies of the HlyA gene in a sample are amplified first by ligase chain reaction (LCR). The products of LCR are then assessed for copies of the HlyA gene using the immobilised probe-primer of Example 1 which becomes extended and labelled in the presence of the gene.

Oligonucleotides corresponding to portions of the coding sequence of the Listeria monocytogenes HlyA gene are prepared using standard techniques of oligonucleotide synthesis. Three primary probes have base sequences corresponding to residues

-27-

757 to 787, 788 to 859 and 860 to 902 of the HlyA gene respectively. Three secondary probes are also prepared. Each secondary probe has a base sequence which is complementary to a respective one of the primary probes. The probe-primer sequence immobilised on the surface of the nylon membrane is prepared as in Example 1 except that it is only 20 bases long and corresponds to residues 778 to 797 of the HlyA gene. The first and second primary probes each have sequence homology with only 10 bases of the immobilised probe-primer. The first primary probe has a 10 base sequence complementary to residues 778 to 787 of the HlyA gene; the second primary probe has a 10 base sequence complementary to residues 788 to 797. Because of this, hybridisation between the first and second primary probes and the probe-primer is unlikely to occur under the hybridising conditions used. Hybridisation takes place preferentially between the primary probes and the complementary strand of the selected portion of the HlyA gene in the sample. The ligation of the three primary primers together during LCR therefore indicates the presence of the complementary strand to the HlyA gene portion. Likewise, the ligation of the three secondary primers together indicates the presence of a strand of DNA carrying the selected portion of the HlyA gene.

The probe-primer is placed in an eppendorf tube with the sample, the three primary probes and the three complementary secondary probes. LCR is performed in accordance with the method described in published European Patent Application No. 0320308-A2.

After completion of LCR a mixture of deoxyribonucleoside triphosphates is added to give the following final concentrations; 200 μ M dATP, 200 μ M dCTP, 200 μ M dGTP, 150 μ M TTP and 50 μ M Biotin-11-dUTP. Three units of Taq polymerase are added and then the tube is incubated at 50°C for 4 minutes to allow hybridisation and subsequent extension of the immobilised probe-primer. The nylon membrane is removed, washed in buffer and assessed for biotin label as in Example

-28-

1.

The addition of the alkaline phosphatase substrate used in Example 1 causes the membrane to become coloured indicating that the avidin carrying the alkaline phosphatase activity is bound to the biotin linked to the membrane. This in turn indicates that the Taq polymerase has extended the immobilised probe-primer in response to the hybridisation of a target sequence to the immobilised probe-primer.

10 Example 3

Labelling extension of an immobilised oligonucleotide in the absence of an amplification process

This example demonstrates how an immobilised oligonucleotide sequence can be extended and labelled without any form of DNA amplification process taking place at the same time.

A probe-primer sequence comprising a homopolymer of dATP 20 nucleotides long is prepared and then bound to the surface of cellulose beads. Such beads are commercially available from Pharmacia as oligo dA cellulose. The beads are placed in an eppendorf and suspended in hybridisation buffer containing 1% (w/v) sodium dodecylsulphate (SDS), 1 M NaCl and five-fold concentration of saline-sodium citrate buffer (SSC) [Ref: Sambrook, J., et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1989), p. B.13.] Oligo dT (25-30 nucleotides long) is added to give a final concentration of 1 $\mu\text{g}.\text{ml}^{-1}$. The mixture is incubated for 30 minutes at 37°C in the presence of a nucleotide mixture having final concentrations of 200 μM dATP 200 μM dTTP, 200 μM dCTP, 200 μM dGTP and 50 μM Biotin-11-dUTP, and 2 units of Klenow enzyme (Gibco-BRL). After incubation the beads are spun down and the reaction mixture removed. The beads are washed twice by resuspending and spinning down in 10 mM Tris-HCl buffer at pH 7.0. After washing the beads are subjected to a colorimetric detection procedure for biotin as in Example 1.

-29-

The beads become coloured indicating that the avidin carrying the alkaline phosphatase enzyme is bound to biotin linked to the membrane. This in turn indicates that biotinylated uridine residues have been incorporated by Klenow polymerase into the extended portion of the immobilised probe-primer.

Example 4

10 Capture and surface labelling following lysis of bacteria

In this example is tested for the presence of copies of the *Listeria monocytogenes* ribosomal 16S RNA gene, the sequence of which is described in PCT/US90.00656.

The probe-primer is constructed to detect the copies of the gene and has the following sequence. (SEQ ID:8)

5'- CCC CCC CCC CCC CCC AAG CAG TTA CTC TTA TCC TTG TTC TT -3'

|
nuc position 448

|
nuc position 472

20 The probe was attached by its 5' end, as described in Maniatis, T, Fritsch, E F, and Sambrook, J(1982), and Ghosh, S S, Musso G F,(1987), to plastic spheres (Polybead Amino Microspheres, 1.0 μ m diameter, catalogue number 17010). An overnight culture of *Listeria monocytogenes* was collected by

25 centrifugation and resuspended to a concentration of 10^6 bacteria/ml. 0.1 ml of this suspension was lysed by heating to 110°C for 5 minutes. A 0.05 ml sample of the lysate was added to 0.05 ml of labelling buffer (final concentration 50 mM Tris-HCC (pH 8.3), 40 mM KCl, 6mM $MgCl_2$, 1mM DTT, 0.5mM dATP

30 (biotinylated) 0.5mM dCTP, dGTP, dTTP). 1 unit of M-MLV reverse transcriptase (Gibco-BRL) was added to the mixture and the reaction was incubated for 30 minutes at 37°C.

Following the labelling period the beads were washed in 0.1%SDS and 2 x SSC for 5 minutes at 90°C. The washing

35 process was repeated using 2 x SSC (without SDS) at 60°C for 5 minutes.

-30-

The beads were then subjected to a standard colorimetric detection procedure for biotin using avidin linked to alkaline phosphatase. Addition of the substrate 2,2' - azido-bis (3 ethyl benzthiozoline -6- sulphonic acid) caused the membrane to become coloured indicating that the avidin carrying the alkaline phosphatase enzyme is immobilised to the beads by binding to biotin. The results of optical density measurements are shown in Table 1 below:-

Table 1

Optical Density

Control (No DNA) 0.21

Sample 0.56

The presence of biotin shows that there has been extension of the bead bound probe primer by the reverse transcription.

Example 5

Coupling of affinity capture and direct detection of Listeria

Listeria cells were trapped on polystyrene microtitre plates pre-coated with monoclonal antibody specific to Listeria spp. Antibody was diluted to 15 $\mu\text{g ml}^{-1}$ in phosphate-buffered saline, pH 7.4 (PBS) and 100 μl was added to each microtitre plate well. After 3 hours at room temperature (c. 21°) the wells were washed three times with PBS. To minimise non-specific binding, the antibody-coated plates were then blocked by treatment with 3% bovine serum albumin in PBS for 45 minutes at 21°C, 300 μl per well. The plates were then washed five times with PBS containing 0.05% Tween-20 (PBST) and allowed to air-dry for 1 hour.

-31-

L monocytogenes cultures, grown in selective broth, were collected by centrifugation. Cells were resuspended and dilution series made in either PBS or PBST. 100 µl of bacterial suspension was incubated in antibody-coated wells for between 30 minutes and 2 hours at 21°C. Finally, the plates were washed three times with PBST.

The immobilised *Listeria monocytogenes* were subjected to heat treatment (110°C for 5 minutes) in order to lyse the bacteria, this releasing the RNA's into the solution.

A 0.05 ml sample of the lysate was mixed with beads prepared as described in Example 4 (having the capture oligonucleotide on the surface of the beads). The rest of the experiment was performed as described in Example 4.

The detection of a colorimetric reaction demonstrated that the system was capable of detecting the presence of micro-organisms without the need to resort to using amplification techniques. The results of the colorimetric detection are shown in Table 2 below:-

20

Table 2

	<u>Optical Density</u>
Control (No DNA)	0.18
Sample (with lysate)	0.49

25

Example 6

30 Specific Detection of DNA Sequences by Surface-Bound Capture Probe and Labelled Extension

Attachment of Capture Probe to Beads

35

Oligonucleotides are first 5-prime-phosphorylated following the method of Maniatis et al (1982); oligonucleotide

-32-

is incubated with ATP in the presence of T4 polynucleotide kinase at 37°C for 30 minutes in 0.5 M Tris. HCl (pH 7.6) containing 0.1 M MgCl₂, 50 mM dithiothreitol and 1 mM EDTA.

Phosphorylated oligonucleotide is attached to polystyrene microspheres with surface amino groups (Polybead Amino Microspheres, 1 µm diameter; Polysciences Inc.) by incubation overnight in 0.1 M methylimidazole buffer pH 6.0, with 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Ghosh and Musso, 1987).

10

Capture and Extension Reaction

Prior to the reaction the target DNA (144 bp fragment produced by PCR) was denatured for 10 minutes at 94°C. The reaction contained 200 µm of each dNTP (the dATP being biotin-labelled), 6 µl undiluted PCR product (target DNA), 1-10 mg of oligonucleotide-coated beads, PCR buffer and 2 U of Taq polymerase in a 100 µl volume. Annealing of the target sequences with the capture probe proceeded for 10 minutes at 55°C, followed by extension of the probe using labelled dATP for 10 minutes at 70°C. The beads were then incubated in 2 x SSC at 94°C for 5 minutes and washed twice with 2 x SSC. Vacant sites on the beads were then blocked by incubation in PBS containing 0.05% Tween-20 (PBST) and 1% bovine serum albumin for 20 minutes at 21°C. Streptavidin-peroxidase (1 µg ml⁻¹ in PBST) was allowed to react for 20 minutes at 21°C. After four washes with PBST, o-phenylenediamine in 0.05M phosphate-citrate buffer pH5.0 containing 0.012% sodium perborate (0.4 mg ml⁻¹) was added. The chromogenic reaction was halted with 3M HCl and the optical density at 490 nm of the supernatant after removing the beads by centrifugation was measured. A significant increase in colour production from reaction mixes containing target DNA over those that did not was observed.

The results of the colorimetric determination are shown in Table 3:-

-33-

Table 3

		<u>Optical Density</u>
5	Control (No DNA)	0.08
	Sample	0.40

Example 7

10

Specific detection of different concentrations of DNA sequences by surface bound capture probe and labelled extension.

15 A capture probe as used in Example 1 was attached to the surface of polybead microspheres as described in Example 6.

A series of PCR's were set up using a constant number of capture probe coated microspheres and reagents as described in Example 1. However the amount of *Listeria* genomic DNA was varied from sample to sample, the three concentrations being
 20 100ng, 1ng, 1pg. The PCR and the colorimetric detection were carried out as in Examples 1 and 6 respectively.

The following data was obtained:

25	Initial Genomic DNA concentration	OD after 10 cycles	OD after 20 cycles
	100ng	0.25	0.37
	1ng	0.20	0.34
30	1pg	0.17	0.24
	Control	0.18	0.19

OD = optical density

35 Thus using a standard set of PCR conditions an approximate quantitative determination of the amount of genomic starting

-34-

material introduced in a sample may be obtained.

As a modification of the methods described herein for amplifying DNA and detecting the product of said amplification or directly detecting DNA without target amplification, one may detect RNA by first producing cDNA from the target sequence of the RNA and then detecting that cDNA by methods previously described herein. One may use a PCR or LCR procedure to amplify the target cDNA. One may use cycles of hybridisation of target cDNA to immobilised probe primer and polymerase mediated extension of the probe primer followed by dehybridisation to label multiple probe primer sites on the solid support as described above to provide signal amplification without amplification of the free target sequence. A typical procedure in summary would be:-

- 1) RNA extraction;
- 2) production of cDNA using specific primer(s) and reverse transcription;
- 3) capture cDNA using immobilised probe primer(s);
- 4) perform thermal cycling in the presence of polymerase (e.g. Taq) and dNPT's, i.e. cycles of denaturation (e.g. 95°C for 1 minute), annealing (e.g. 55°C for 30 seconds), and extension of the probe primer (e.g. 70°C for 1.5 minutes) followed by detecting probe primer extension.

25

SEQUENCE LISTING

Seq ID:1
length=8 bases
type=nucleotide

AAAATGCT 8

Seq ID:2
length=4
type=nucleotide

GGGG 4

Seq ID:3
length=21
type=nucleotide

TTTTACGTTC TTCTTCAGTA A 21

Seq ID:4
length=41
type=nucleotide

CCCCCCCCCCC CCCCCCCCCC TTTTACGTTC TTCTTCAGTA A 41

Seq ID:5
length=41
type=nucleotide

CCCCCCCCCCC CCCCCCCCCC TTTTACGTTC TTCTTCAGTA A 41

Seq ID:6
length=20
type=nucleotide

TCGGCGCAAT CAGTGAAGGG 20

Seq ID:7
length=20
type=nucleotide

CTCCAAGCGC TTGCAACTGC 20

Seq ID:8
length=41
type=nucleotide

CCCCCCCCCCC CCCCCAAGCA GTTACTCTTA TCCTTGTTCT T 41

Seq ID:9
length=8
type=nucleotide

AGCATTTT

8

Seq ID:10
length=4
type=nucleotide

CCCC

4

Seq ID:11
length=4
type=nucleotide

AAAA

4

-37-

CLAIMS

1. A method for establishing the presence or absence in a sample of a nucleic acid containing a target nucleotide base sequence or quantitating the presence of said nucleic acid
5 containing the target nucleotide base sequence, comprising the steps of:

- 10 (a) exposing a sample of nucleic acid to an immobilised nucleic acid probe-primer, at least a portion of the probe-primer being capable of hybridisation with the target sequence if present in said sample under conditions such as to allow the target sequence if present to hybridise with the immobilised probe-primer;
- 15 (b) (i) subjecting any probe-primer/target sequence hybrids so formed to a polymerase reaction in the presence of at least one labelled deoxynucleoside triphosphate substrate so as to extend and label the probe-primer; or
- 20 (b) (ii) subjecting any probe-primer/target sequence hybrids so formed to hybridisation of a portion of said target sequence adjacent the portion hybridised to said probe/primer, said hybridisation being with a labelled second oligonucleotide primer
25 complementary in sequence to said adjacent portion of the target sequence, and ligating said probe-primer and second primer; and
- 30 (c) detecting or quantitating any label or amount of label bound to the extended immobilised probe-primer.

2. A method for producing immobilised labelled nucleic acid comprising the steps of:-

- 35 (a) exposing nucleic acid containing a target nucleotide base sequence to an immobilised nucleic acid probe-primer under hybridising conditions, at least a portion of the probe-primer being capable

-33-

of hybridisation with the target base sequence, so as to allow the nucleic acid containing the target base sequence to hybridise with the probe-primer; and

- 5 (b) (i) subjecting the hybridised probe-primer to a polymerase reaction in the presence of at least one labelled deoxynucleoside triphosphate substrate so as to extend and label the probe-primer; or
- 10 (b) (ii) subjecting any probe-primer/target sequence hybrids so formed to hybridisation of a portion of said target sequence adjacent the portion hybridised to said probe/primer, said hybridisation being with a labelled second oligonucleotide primer complementary in sequence to said adjacent portion
- 15 of the target sequence, and ligating said probe-primer and second primer.

3. A method as claimed in Claim 2, wherein after the polymerase reaction the immobilised probe-primer is separated from the nucleic acid containing the target sequence and labelled deoxynucleoside triphosphates.

20 4. A method as claimed in Claim 1 or Claim 3, wherein the immobilised probe primer is separated from the sample and any unreacted deoxynucleoside triphosphate substrates under denaturing conditions or wherein after said separation, any

25 double stranded nucleic acid formed by said probe-primer extension is denatured and the free denaturation products are separated from said immobilised extended probe-primer.

5. A method as claimed in Claim 1 or Claim 2, wherein the polymerase reaction is carried out in the presence of

30 denatured nucleic acid complementary in sequence to said nucleic acid containing said target sequence.

6. A method as claimed in Claim 5, wherein during said polymerase reaction two oligonucleotide primers are provided, the first primer being capable of hybridisation to a

35 complementary strand at a location which lies in the 3' direction from the 3' end of the sequence of the complementary

strand which is complementary to the target sequence to which the immobilised probe-primer hybridises, the second primer being capable of hybridisation with said target sequence containing nucleic acid at a location which lies in the 3' direction from the 5' end of the target sequence to which the probe-primer hybridises and the first primer having no sequence homology with the immobilised probe primer or the said second primer sufficient to produce hybridisation therebetween.

7. A method as claimed in Claim 6, wherein the nucleotide base sequence of the second primer is the same as at least a portion of the nucleotide base sequence of the immobilised probe-primer.

8. A method as claimed in Claim 7, wherein the immobilised probe-primer is longer than the said second primer.

9. A method as claimed in any preceding claim, comprising performing multiple cycles of denaturing the product of the polymerase extension performed in step (b)(i) or the ligation performed in step (b)(ii) to liberate the sample nucleic acid therefrom, rehybridising said sample nucleic acid to further immobilised probe-primer and re-conducting steps (b)(i) or (b)(ii) prior to step (c).

10. A method as claimed in any preceding claim, wherein nucleic acid exposed to the immobilised probe-primer is a product of a polymerase chain reaction (PCR) process or ligase chain reaction (LCR) process.

11. A method as claimed in Claim 10, wherein at least one amplification cycle of a PCR process is conducted in the presence of said probe-primer.

12. A method as claimed in any preceding claim, wherein the polymerase reaction is carried out using one or more of Taq polymerase, DNA polymerase I, DNA polymerase Klenow fragment, or T4 polymerase.

13. A method as claimed in any preceding claim, wherein the probe-primer comprises between 20 and 40 nucleotide bases.

14. A method as claimed in any preceding claim, wherein the

-40-

immobilised probe-primer is covalently linked to a solid support in such a way as to leave its 3' end available for extension.

5 15. A method as claimed in any preceding claim, wherein the label is a radioisotope, is biotin or is an enzyme.

16. A method as claimed in Claim 15, wherein the label is detected by fluorescence, radiography, scintillation counting or enzyme catalysed assay reaction.

10 17. A method as claimed in Claim 15, wherein a biotin label linked to the probe-primer is determined by washing with an avidin-enzyme complex followed by washing away any unbound avidin-enzyme complex and then incubating with a suitable enzyme substrate to produce a detectable product.

15 18. A method as claimed in Claim 15, wherein a biotin label linked to the probe-primer is determined by washing with an avidin-fluorescent molecule conjugate, washing away any unbound avidin-fluorescent molecule conjugate and then viewing under a light source of appropriate wavelength.

20 19. A method of detecting the presence in a sample of copies of a target nucleic acid sequence generated by a polymerase chain-reaction process comprising:

25 (a) exposing an immobilised probe-primer sequence complementary to at least a part of the target sequence to a denatured sample under hybridising conditions;

30 (b) subjecting the immobilised probe-primer to a polymerase reaction in the presence of at least one labelled deoxynucleoside triphosphate substrate so that the immobilised probe-primer becomes extended and labelled when hybridised to a portion of the target sequence; and

(c) detecting any label bound to the immobilised probe-primer.

35 20. The use of a nucleic acid probe-primer immobilised to a solid support for hybridisation and extension in a polymerase reaction utilising at least one labelled nucleotide.

-41-

21. A method of detecting the presence in a sample of copies of a target nucleic acid sequence generated by a polymerase chain-reaction process employing a first primer and a second primer comprising:

- 5 (a) conducting at least one PCR cycle in the presence of an immobilised probe-primer sequence complementary to at least a part of the target sequence in addition to said first and second PCR primers so that the immobilised probe-primer
10 becomes extended when hybridised to a portion of the target sequence; and
(b) detecting the presence of the extended immobilised probe-primer.

22. A method as claimed in Claim 21, wherein the said at
15 least one PCR cycle is carried out in the presence of at least one labelled deoxynucleoside triphosphate substrate so that the probe-primer becomes labelled as it is extended.

23. A method as claimed in Claim 22, further comprising
20 separating the extended immobilised probe-primer from the sample and any unreacted labelled deoxynucleoside triphosphate and detecting the presence of the label in the extended immobilised probe-primer.

24. A method as claimed in Claim 21, comprising in step (b)
25 denaturing extension products of the probe-primer and hybridising a labelled probe to the bound extension products.

25. A method of detecting a target nucleic acid sequence comprising the polymerase or ligase mediated extension and labelling of an immobilised probe-primer sequence complementary to at least a part of the target sequence in the
30 presence of products of a polymerase chain reaction (PCR) or a ligase chain reaction (LCR), at least a portion of the base sequence of the said PCR or LCR products containing the target sequence.

26. A kit for performing a method as claimed in any one of
35 Claims 1 to 25, comprising a nucleic acid probe-primer immobilised to a solid support such that its 3' end is

-42-

available for polymerase extension, and one or more of; a DNA
polymerase enzyme, appropriate nucleoside triphosphate
substrates at least one of which is labelled, one or more
oligonucleotide primers for amplification of a nucleic acid
5 sequence complementary to that of said probe-primer, a DNA
ligase enzyme, or a labelled oligonucleotide probe for a
portion of said target sequence adjacent the portion of said
sequence which is complementary to said probe-primer.

10

1/3

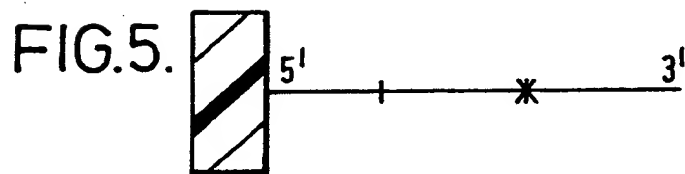
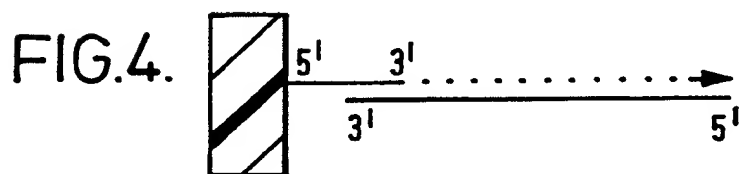
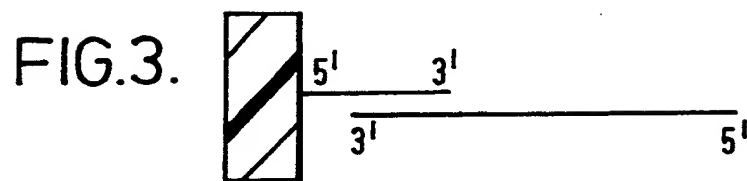
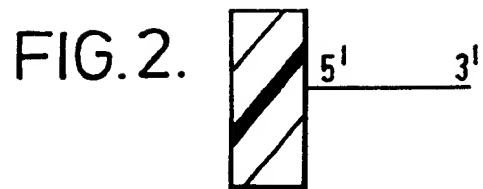
1. Substrate - poly C linker - 5' 3'
AAATGCT
2. 3' 5'
TTTACGAXXXX-XXXXGGG
3. 5' 3'
AAATGCTYYYY-YYYYCCCC
4. 3' 5'
GGG
5. 5' 3'
AAAA
6. 3' 5'
ZZZZ-ZZZZTTTACGAXXXX-XXXXGGGGZZZZ-ZZZZ
7. 5' 3'
ZZZZ-ZZZZAAATGCTYYYY-YYYYCCCCZZZZ-ZZZZ

1. = immobilised probe primer
2. = PCR product strand 1 (target for immobilised probe-primer)
3. = PCR product strand 2
4. = first PCR primer
5. = second PCR primer
6. = PCR target nucleic acid strand 1
7. = PCR target nucleic acid strand 2

FIG.1.

SUBSTITUTE SHEET

2/3



SUBSTITUTE SHEET

3/3

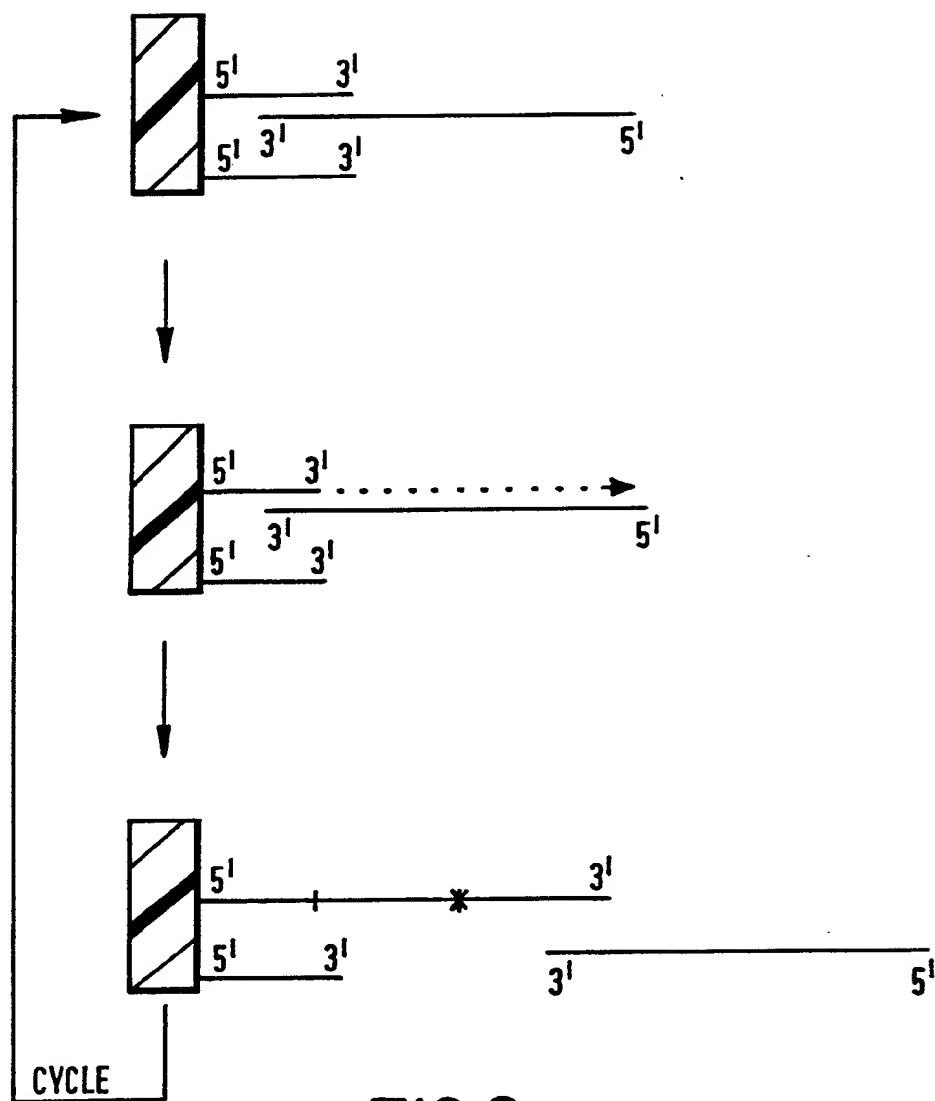


FIG.6.

SUBSTITUTE SHEET